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Genetic reprogramming for NK cell cancer immunotherapy with CRISPR/Cas9

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Summary

Natural killer cells are potent cytotoxic lymphocytes specialized in recognizing and eliminating transformed cells, and in orchestrating adaptive anti-tumour immunity. However, NK cells are usually functionally exhausted in the tumour microenvironment. Strategies such as checkpoint blockades are under investigation to overcome NK cell exhaustion in order to boost anti-tumour immunity. The discovery and development of the CRISPR/Cas9 technology offer a flexible and efficient gene-editing capability in modulating various pathways that mediate NK cell exhaustion, and in arming NK cells with novel chimeric antigen receptors to specifically target tumour cells. Despite the high efficiency in its gene-editing capability, difficulty in the delivery of the CRISPR/Cas9 system remains a major bottleneck for its therapeutic applications, particularly for NK cells. The current review discusses feasible approaches to deliver the CRISPR/Cas9 systems, as well as potential strategies in gene-editing for NK cell immunotherapy for cancers.

Keywords: checkpoint; delivery; genetic manipulation; NK dysfunction; off-target.

Introduction

Natural killer (NK) cells, as the first line in host immune surveillance, play an essential role in tumour immunity.[1] However, NK cells are usually functionally exhausted in the tumour microenvironment, and thus preventing them from fully displaying their anti-tumour potential.[2] Besides, NK cells, unlike T-cells, lack the antigen-specific recognition capability. Over the years, approaches such as administration of cytokines or antibodies, or gene-editing technologies, have been investigated to overcome the immune suppression in tumours, or to enhance tumour target recognition in NK cell immunotherapy.[3,4] The recent development of flexible and efficient CRISPR/Cas9 genome-editing technologies has offered new opportunities in this area.[5,6] Although *in situ* genomic editing of NK cells *in vivo* is still difficult to achieve, genetic engineering of *ex vivo* NK cells for adoptive therapy, or *in situ* genetic engineering of tumours *in vivo* to increase their susceptibility to NK surveillance, has been much easier and represents promising areas to explore.

NK cells are anti-tumour effector cells usually dysfunctional in the tumour microenvironment

Natural killer cells are innate immune lymphocytes that play anti-tumour and anti-viral functions, either through direct cytotoxic activity or production of effector cytokines.[1] Activating NK cells involves a balance of activating and inhibitory signals from the surface of NK cells upon target cell recognition.[7] Based on this balance, they recognize stressed, transformed or infected cells through target cell surface molecular patterns.[7] Upon target cell recognition, NK cells eliminate target cells through cytotoxicity

Abbreviations: CAR, chimeric antigen receptor; Cas, CRISPR-associated protein; CRISPR, clustered regularly interspaced short palindromic repeat

by releasing granzyme and perforin to the target cell, or through FasL, TNF-α and TRAIL, leading to a cellular event involving DNA fragmentation and apoptosis of target cell.[1] Upon activation, NK cells also produce cytokines such as IFN-γ to facilitate the responses of the adaptive immunity[8,9] and to directly modulate the architecture of the tumour microenvironment.[10] Furthermore, due to potentially less severe side-effects and fewer costs compared with chimeric antigen receptor (CAR)-T-cells, NK cells have emerged as a safe and effective alternative as CAR-modified immune cells for cancer immunotherapy.[11,12]

Despite the effector potential of NK cells, they are usually dysfunctional in the tumour microenvironment.[13–15] In addition, tumour-associated NK cells undergo differentiation to type 1 innate lymphoid cells (ILC1) with decreased anti-tumour effector functions, thus losing control of tumour growth.[16,17] Studies have proven the concept of, and have shown the potentials of, targeting some pathways for the reinvigoration of NK cell anti-tumour immunity.[3,4,18] To target pathways that mediate NK cell dysfunction, as well as to enhance NK cell-activating pathways, the CRISPR/Cas9 gene-editing technology offers a powerful tool to employ.

The CRISPR/Cas9 system and its delivery

The concept of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats, and CRISPR-associated proteins) technology originates from the prokaryotic adaptive immune system, which provides bacteria with resistance to foreign nucleic acids.[19] With the rapid development of this technology, designs can be made for recognition of specific loci in the genome to create DNA double-strand breaks (DSBs), which leads to repair and subsequent gene knock-out/knock-in or forced expression.[20]

In order to apply the CRISPR/Cas9 technology for gene-editing, the Cas9 protein and target-site-specific gRNA, or their encoding nucleic acid, need to be delivered into the cells. In some cases where gene knock-in is intended, a donor DNA with homology to the sequences flanking the DSB location is required in such a situation. NK cells, particularly primary NK cells, are well known to resist ordinary transfection, making the delivery of the Cas9 system difficult, and thus representing an important concern in gene-editing of NK cell immunotherapy. To achieve the delivery, integrating (lentivirus and retrovirus)[21] or non-integrating (adenovirus and adeno-associated virus)[22–24] transduction are both applicable approaches. Among these viral vectors, adeno-associated virus is currently the preferred vector for CRISPR/Cas9 system components delivery for immunotherapy, and is usually used for *in vivo* somatic gene delivery due to their low immunogenicity.[25] Aside from transduction, electroporation with Cas9 (protein or encoding DNA/mRNA) and gRNA (whether chemically modified or not) is another feasible approach to deliver the system into the cells, especially in situations where stable genomic integration of CRISPR/Cas9 components is not favourable, avoiding cellular toxicity, rejection by host adaptive immunity, and regulatory concerns in clinical settings.

Although *ex vivo* lymphocyte gene-editing by CRISPR/Cas9 has been widely reported, *in vivo* CRISPR/Cas9 gene-editing is still challenging. In addition to viral delivery, emerging nanomaterials-based delivery systems might be a possible direction due to the flexibility in modifications of the materials, and some preliminary studies already displayed their potential. For example, cationic α-helical PEGylated polypeptide nanoparticles were shown to be efficiently uptaken by cells for delivery of Cas9 expression plasmid and sgRNA both *in vitro* and *in vivo* for gene deletion.[26] However, in order to achieve gene knock-in by homology-directed repair, donor DNA also needs to be delivered into cells in addition to Cas9 protein and gRNA, therefore demanding higher loading capability of the vehicle. To this end, the features of gold nanoparticles to be coated with protein and a dense layer of DNA was applied in one study.[27] In this study, gold nanoparticles were conjugated to thiol-modified oligonucleotides, which were hybridized with single-stranded donor DNA.[27] This was then complexed with Cas9 ribonucleoprotein and cationic endosomal disruptive polymers.[27] The cationic polymers led to the internalization of this CRISPR-Gold by cells via endocytosis, and triggered endosomal disruption, resulting in the release of Cas9 ribonucleoprotein and donor DNA into the cytoplasm for editing.[27] Local injection of this CRISPR-Gold was shown to efficiently correct the DNA mutation that causes Duchenne muscular dystrophy in mice.[27] Besides nanomaterials-based delivery systems, efforts have also been made to engineer Cas9 with multiple SV40 NLS (nuclear localization sequences) to possess innate cell-penetrating capabilities, and to enable efficient gene-editing *in vivo* through direct local delivery of the Cas9 ribonucleoprotein complexes, shedding light on the potential for engineering the Cas9 protein specifically for *in vivo* delivery.[28] On the other hand, cell-type-selective delivery method has been reported, which was mediated by interactions between receptor and its specific ligand.[29] For example, Cas9 proteins harbouring asialoglycoprotein receptor ligands increased the accumulation in HepG2 cells, which were highly expressing the asialoglycoprotein receptor, compared with control SKHEP cells, which express low levels of this receptor,[29] possibly due to asialoglycoprotein receptor-mediated endocytosis. Although the *in vivo* efficiency remains unclear, it represents an ideal strategy for precise delivery of the Cas9 materials worthy of further investigations.

Although NK cells are relatively resistant to viral transfection, efficient CRISPR/Cas9-mediated genome-editing of human NK cells has been reported via electroporation

of Cas9 ribonucleoprotein complexes by a Lonza 4D nucleofector system.[30,31] In one study, electroporation of primary human NK cells with Cas9 ribonucleoprotein in P3 Primary Cell Nucleofector™ solution using pulse-code 'EN-138' resulted in proportional indel rates of about 30%, and a 60% reduction in *Tgfbr2* mRNA expression level, rendering NK cells more resistant to TGF-β *in vitro*.[30] This study also indicates that the gene-editing by electroporation of the Cas9 ribonucleoprotein complexes was feasible. On the other hand, poor survival post-electroporation is another hurdle for NK cell gene-editing via electroporation. Another study therefore performed extensive optimization on buffers and pulse-codes, and found that pulse-code 'CM-137' with a mannitol-supplemented phosphate buffer (5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 150 mM Na_2HPO_4/NaH_2PO_4, 50 mM mannitol, pH 7.2) was optimal, with >80% NK cell viability and uptake of large molecule FITC-Dextran mimicking the Cas9 complex, with observed 45–80% indel rates in primary human NK cells.[31] Interestingly, by using this approach, this study generated human NK cells with the targeted gene *Ptprc* either totally lost, unaffected or at intermediate levels, suggesting that homo- and heterozygous human NK cells can be generated.[31] Therefore, with the optimized parameters for both high viability and efficiency, electroporation of Cas9 ribonucleoprotein represents a promising approach for genetic modification of human NK cells for adoptive immunotherapy.

Genetic reprogramming strategies in NK cell immunotherapy

As discussed above, multiple pathways are implicated in NK cell anti-tumour immunity. In order to fully exploit the anti-tumour potential of NK cell immunotherapy, we might need to simultaneously enhance some, while suppressing other, pathways. To this end, the multiplex capability of the CRISPR/Cas9 system perfectly fits this requirement. CRISPR/Cas9 offers the ease of site-specific integration of gene of interest (with donor gene through homology-directed repair pathway), while concurrently deleting multiple genes of interest. The following sections will focus on existing and potential gene-editing strategies, where CRISPR/Cas9-based genetic modification is applicable, to improve NK cell tumour surveillance by enhancing tumour recognition, activation, infiltration and persistence, and by antagonizing inhibitory pathways (Fig. 1).

Arming with CARs

In order to improve specific recognition of tumour surface markers, NK cells can be armed with CARs by gene-editing. CARs consist of an extracellular single-chain variable fragment (ScFv) specific to a tumour antigen, and an intracellular signalling domain that transduces activation signalling upon antigen recognition. NK cells armed with CARs targeting tumour antigens have displayed antigen-specific anti-tumour effects *in vitro* and *in vivo*,[32–36] indicating that specific recognition of tumour antigen by NK cells could be achieved by arming with CARs.

On the other hand, arming with pan-specific CAR-like molecules represents another attractive strategy to improve tumour recognition. Because tumour evasion through losing the original tumour antigen is currently a challenge to CAR-based immune cell therapies, the multiple-ligands recognition mode by some NK cell receptors might be superior in tumour recognitions, possibly leading to more durable anti-tumour responses. For example, human NKG2D ligands, including MICA and MICB (MHC class I chain-related proteins A and B), and six ULBPs (UL16-binding proteins), are expressed poorly or not at all by most normal cells, but are upregulated in tumour cells and virus-infected cells.[37] Recognitions of more than a single tumour marker would likely improve tumour recognition compared with conventional single CARs. The fusion of full-length NKG2D protein with CD3ζ, as a combination of the concept of CAR design with the multiple-ligands recognition feature of NKG2D, exhibits potent efficacy against NKG2D ligand-positive tumours when expressed on T-cells or NK cells.[38,39] Additionally, this method potentially antagonizes the immunosuppressive tumour microenvironment by targeting NKG2D ligand-positive MDSCs and Tregs in tumours.[38]

While the extracellular domain of CARs should be designed based on the best recognition of the tumours to target, the design for intracellular domain of NK-CARs might be different from that for T-cells, and should be optimized specifically for NK cells. Existing reports showed that among many potential signalling domains for CAR-NK design, when used alone, only DAP12 could show improved activating effects when compared with CD3ζ.[40,41] When used in combination with CD3ζ, DAP10,[42]B4[32] and 4-1BB[43] demonstrated enhanced triggering capabilities compared with CD3ζ alone. It is interesting that only DAP12 showed CD3ζ-independent and CD3ζ-comparable triggering effects, which suggest that DAP12 might play a predominant role in activating signalling in NK cells. Future studies need to test whether DAP12 in combinations with other signalling domains could display even prominent triggering effects in CAR-NK design.

Enhancing activating pathways

The responses of NK cells could be triggered and/or sustained by activating cytokines (such as IL-2, IL-15, IL-18 and IL-21) and signalling through activating receptors,

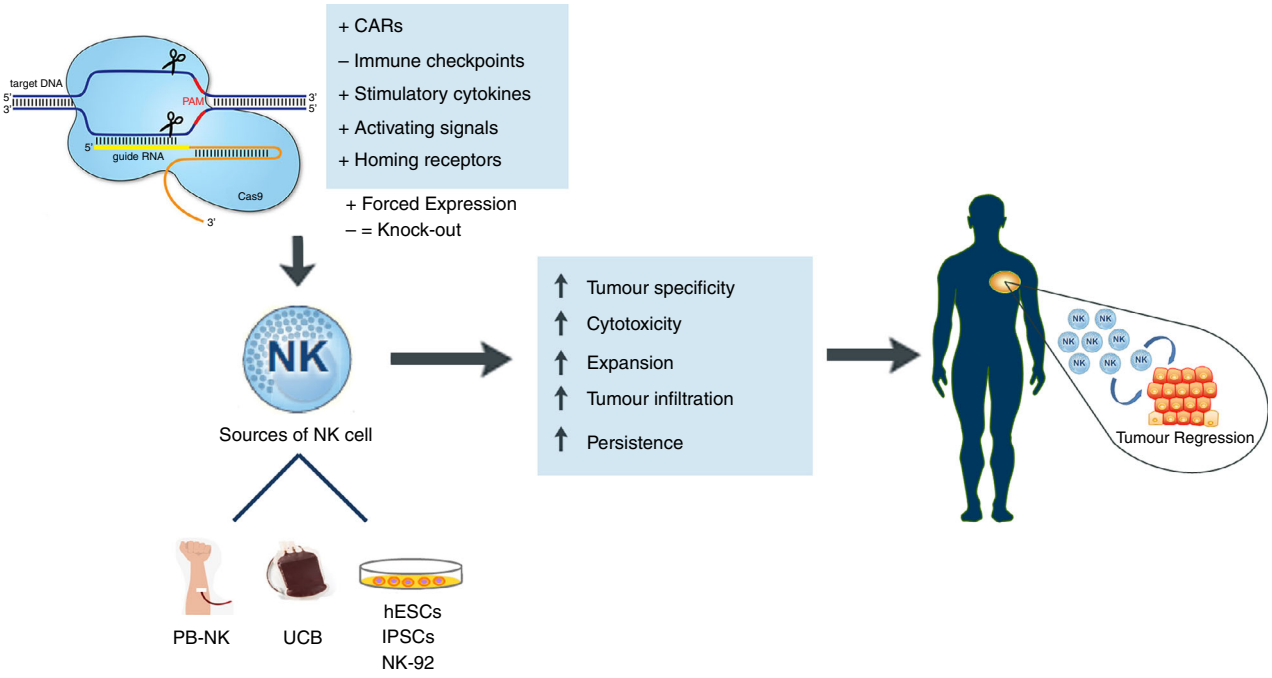

Figure 1. Schematic overview of the CRISPR/Cas9-mediated genetic reprogramming of NK cells for cancer immunotherapy. Genetic editing of NK cells from various sources (PBNK: Peripheral Blood NK cells; UCB: Umbilical Cord Blood; hESCs: haematopoietic Embryonic Stem Cells; iPSCs: induced Pluripotent Stem Cells; NK-92: NK-92 cell line) via the CRISPR/Cas9 multiplexing by knocking out immune checkpoints and inhibitory signals, bolstering its tumour infiltration by integrating tissue homing receptors, improving its anti-tumour activity by providing extra activating signalling, as well as enhancing its tumour specificity by arming with chimeric antigen receptors (CARs).

which have been investigated for NK cell immunotherapy.[4] Among these factors, IL-15 and IL-2 are most well established in their survival-promoting effects on NK cells. Cord blood-derived NK cells transduced to express IL-15 showed marked prolongation of survival in a xenograft Raji lymphoma murine model.[44] On the other hand, 'Super-2', a mutant form of IL-2, binds IL-2Rβ with high affinity, promotes NK cell proliferation, and reverses the exhausted state of NK cells without evoking the expansion of Treg cells.[45] Therefore, overexpression of cytokines like IL-15 or 'Super-2' in NK cells for adoptive transfer might potentially render NK cell adoptive transfer therapy with more efficacy.

Apart from gene-editing of NK cells for adoptive transfer therapy, enhancing the *in situ* expression of tumour-derived ligand(s) for NK cell-activating receptors represents another feasible strategy to trigger NK cell anti-tumour responses by enhancing the activating pathways, which could potentially be achieved by CRISPR/Cas9-mediated transcriptional activation. For instance, expression of NKG2D ligand, MICA, has been transcriptionally activated successfully by CRISPR/Cas9.[46]

Improving NK cell infiltration

Tumour infiltration of NK cells in cancer patients is associated with good prognosis in multiple tumours.[47–51] The importance of migration and homing of NK cells to disease sites is critical to its success for cell-based immunotherapy upon adoptive infusion. The enhancement of tumour-specific chemokine receptor expression on NK cells may benefit NK cell immunotherapy in this regard, where CRISPR/Cas9 technology is applicable. Intra-tumoural infiltration of one NK cell subpopulation in tumours of renal cell carcinoma patients correlates with high concentrations of CXCR2 ligands.[52] Genetic engineering of human NK cells to express CXCR2 has been shown to improve their ability to specifically migrate along a chemokine gradient of recombinant CXCR2 ligands *in vitro*, as well as migration to renal cell carcinoma in the mouse model.[52] This indicates that CXCR2 represents a potential chemokine receptor that can be overexpressed on NK cells for improving infiltration into tumours. On the other hand, the CCL-19-CCR7 axis has also been shown to promote immune cell infiltration.[53] NK cells engineered with the chemokine receptor CCR7 displayed significant migratory ability towards its ligands CCL-19 and CCL-21, with improved tumour homing.[54,55] Further analysis of the chemokine signatures of tumour microenvironment might aid in the design of the optimal chemokine receptors profile for improved therapeutic NK cell infiltration into tumours.

On the other hand, the chemokine-driven infiltration of NK cells into tumours is inhibited by the stroma in

the tumour tissues. Therefore, the ability to penetrate tumour-associated stroma is critical for therapeutic NK cell infiltration into tumours. To this end, forced expression of the enzyme heparanase, together with specific CARs, was shown to render CAR-T-cells with improved capacity to degrade the ECM, which promoted T-cell infiltration and anti-tumour activity.[56] Heparanase is also essential for NK cell infiltration of primary tumours, as well as its recruitment to sites of metastasis.[57] Forced expression of heparanase might benefit adoptive NK cell penetration into tumour tissues.

Targeting inhibitory pathways

Detrimental modulations of checkpoint receptor signalling were proposed to mediate NK cell functional exhaustion in tumours.[2] Blockade of checkpoint receptors CD96, TIGIT or NKG2A was shown to boost NK cell anti-tumour immunity.[58–60] In addition to these checkpoint cell-surface receptors, emerging immunosuppressive pathways contribute to the hypofunctional status of tumour-associated NK cells. Genetic disruption of these pathways therefore might improve the effector functions of NK cells for adoptive therapy. For example, PD-1 disruption by CRISPR/Cas9-enhanced CAR-T-cell effector functions.[61–63] In another study, CRISPR/Cas9-mediated targeting of checkpoint molecule *Cish* gene enhanced the cytotoxicity of primary human NK cells towards Daudi B lymphoma cells *in vitro*.[31] Furthermore, genetic targeting of another immunosuppressive pathway, adenosine 2A receptor, enhanced CAR-T-cell efficacy.[64]

Current challenges and future perspectives

With the advances in CRISPR-based genome-editing, most concerns are on the off-target effects causing undesired modifications to the genomic nucleic acids. Efforts have been made to improve the nuclease specificity and precise delivery of the CRISPR/Cas9 system,[65] as well as to develop strategies for superior gRNA design and off-target validation to minimize undesired genome-editing effects.[66]

Furthermore, although the *in vivo* delivery of CRISPR/Cas9 components represents greater therapeutic opportunities, the *in vivo* adaptation of current delivery approaches for CRISPR/Cas9, as well as for other gene-editing technologies, are limited by both the low delivery efficiency and the lack of cell type specificity. Future studies are required to develop novel vehicles or modified versions of the Cas9 enzyme to fully exploit the *in vivo* therapeutic potentials of the CRISPR/Cas9 gene-editing technologies.

In addition to technical barriers, the biggest challenge in NK cell immunotherapy for cancers is the lack of in-depth understanding of the underlying mechanisms of NK cell dysfunction, not only in tumours, but also during *ex vivo* expansion before adoptive transfer.[67] A better understanding of NK cell basic biology, as well as of the tissue-specific features of tumour-infiltrating NK cells, would reveal more NK-specific 'checkpoints' and triggering pathways, and would benefit rational designs for NK cell immunotherapy for cancers with higher efficacy.

In conclusion, the CRISPR/Cas9 system has shown enormous potential in genomic-editing to enhance NK cell immunotherapy, by arming NK cells with CAR constructs, enhancing NK activating pathways, facilitating NK infiltration into tumours, and by antagonizing inhibitory pathways. With future efforts and advances in limiting the off-target effects of this system, increasing *in vivo* delivery efficiency, and ideally achieving cell-type-directed delivery, the CRISPR/Cas9 system should be able to fully potentiate NK cell immunotherapy.

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Disclosures

The authors declare no competing financial interests.

Author contributions

LA and JB conceived the idea and wrote the manuscript. AA and MS made critical comments on the manuscript. XW and JB oversaw the writing process.

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